Phosphatidylinositol 3-Kinase Activation Attenuates the TLR2-Mediated Macrophage Proinflammatory Cytokine Response to Francisella tularensis Live Vaccine Strain

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*J Immunol* published online 22 November 2010

http://www.jimmunol.org/content/early/2010/11/19/jimmunol.0903790
Phosphatidylinositol 3-Kinase Activation Attenuates the TLR2-Mediated Macrophage Proinflammatory Cytokine Response to *Francisella tularensis* Live Vaccine Strain

Edward A. Medina,*† Ian R. Morris,* and Michael T. Berton*

An inadequate innate immune response appears to contribute to the virulence of *Francisella tularensis* following pulmonary infection. Studies in mice suggest that this poor response results from suppression of proinflammatory cytokine production early during infection, but the mechanisms involved are not understood. PI3K is known to regulate proinflammatory cytokine expression, but its exact role (positive versus negative) is controversial. We sought to clarify the role of PI3K in regulating proinflammatory signaling and cytokine production during infection with *F. tularensis* live vaccine strain (LVS). In this study, we demonstrate that the induction of TNF and IL-6 expression by LVS in mouse bone marrow-derived macrophages was markedly enhanced when PI3K activity was inhibited by either of the well-known chemical inhibitors, wortmannin or LY294002. The enhanced cytokine expression was accompanied by enhanced activation of p38 MAPK and ERK1/2, both of which were critical for LVS-induced expression of TNF and IL-6. LVS-induced MAPK activation and cytokine production were TLR2- and MyD88-dependent. PI3K/Akt activation was MyD88-dependent, but was surprisingly TLR2-independent. LVS infection also rapidly induced MAPK phosphatase-1 (MKP-1) expression; PI3K and TLR2 signaling were required. Peak levels of MKP-1 correlated closely with the decline in p38 MAPK and ERK1/2 phosphorylation. These data suggest that infection by LVS restraints the TLR2-triggered proinflammatory response via parallel activation of PI3K, leading to enhanced MKP-1 expression, accelerated deactivation of MAPKs, and suppression of proinflammatory cytokine production. This TLR2-independent inhibitory pathway may be an important mechanism by which *Francisella* suppresses the host's innate immune response. *The Journal of Immunology*, 2010, 185: 900–9000.

*Francisella tularensis* is a Gram-negative, facultative, intracellular coccobacillus that causes the potentially fatal disease tularemia. There are four subs., subsp. *tularensis* (type A), subsp. *holarctica* (type B), subsp. *novicida*. Subsp. *tularensis* and subsp. *holarctica* are the two subspecies that cause clinically significant infection in humans. *F. tularensis* live vaccine strain (LVS) is a live, attenuated vaccine strain developed from the subsp. *holarctica* strain. Whereas LVS in humans causes minimal disease, infection in mice is lethal and mimics human tularemia (1, 2). Recent studies suggest that an inadequate innate immune response to infection with *F. tularensis* likely contributes to its extreme virulence in humans and mice. Indeed, proinflammatory cytokines such as TNF, IL-12, and IFN-γ appear crucial for effective immunity to and survival of *F. tularensis* infection (3–6), yet infection itself appears to attenuate or delay the expression of these critical cytokines (7–10). The mechanism(s) by which *F. tularensis* infection delays or impairs the proinflammatory response remain unknown.

Recent studies have demonstrated that *F. tularensis* triggers the innate immune response via TLR2 (11–16). TLRs are pattern recognition receptors (PRRs) that are evolutionarily conserved and germline encoded. Currently, 10 functional TLRs are known in humans and 12 in mice (17, 18). TLRs bind pathogen-derived molecules and trigger intracellular signals that lead to chemokine and proinflammatory cytokine production, activation of innate immune effector mechanisms, and the upregulation of MHC and costimulatory molecules on APCs, all of which influence the development and nature of adaptive immunity (19). TLRs signal via interactions with four known cytoplasmic adapter proteins: MyD88, Toll/IL-1R domain-containing adapter protein/MyD88 adaptor-like (TIRAP/Mal), Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF), and TRIF-related adapter molecule (TRAM). All the known TLRs, except for TLR3, signal through MyD88, and TLR2 and TLR4 recruit MyD88 via TIRAP/Mal. TLR4 can also use the TRIF and TRAM adaptors to generate signals independently of MyD88. TLR3 appears to signal exclusively through TRIF. The adaptor proteins subsequently recruit and activate the IL-1R-associated kinases 4 and 1 and TNFR-associated factor 6 or TNFR-associated factor 3, leading to the activation of NF-κB and MAPKs and the expression of proinflammatory cytokine genes (20–22). A critical role for PRRs in the innate immune response to *F. tularensis* was evident in an early study that showed that MyD88−/− mice died rapidly when infected with a sublethal dose of LVS (23). Infection with *F. tularensis* has since been demonstrated to induce the innate
inflammatory cytokine and chemokine response via TLR2, and mouse survival is critically dependent on TLR2 expression (11–13, 15, 16, 24). Indeed, macrophages and dendritic cells from TLR2−/− mice make poor proinflammatory cytokine responses to F. tularensis, and mice deficient in TLR2, but not TLR4, are much more susceptible to infection and death than wild-type (WT) controls (13, 16, 25). Interestingly, TLR2-dependent proinflammatory cytokine responses in the lung are significantly delayed (7, 8), and several in vitro studies have suggested that F. tularensis can directly inhibit proinflammatory signaling in infected macrophages and dendritic cells (7, 9, 10, 26). Although progress has been made in understanding the roles of TLRs and other PRRs (27–29) in recognizing F. tularensis, little is known about how F. tularensis modulates PRR signaling to its advantage during infection.

The PI3K/Akt pathway has emerged as an important regulator of innate immune responses. Several TLRs have been reported to activate the class IA PI3Ks (30–35), which are comprised of a regulatory subunit (p85) and a catalytic subunit (p110). The p110 subunit phosphorylates phosphoinositides, leading to the activation of the serine/threonine kinase Akt (36). Certain TLRs appear to interact directly with the p85 subunit to activate PI3K (30, 32); others, such as TLR4, TLR5, and TLR9 require MyD88 to activate the lipid kinase (31, 33–35). TLR-induced activation of the PI3K/Akt pathway regulates diverse functions in inflammatory cells, including phagocytosis (37, 38), inflammatory cell migration (39), apoptosis in mast cells (40), and NO synthase expression in macrophages (41). However, the role of the PI3K/Akt pathway in regulating TLR-induced cytokine production in response to microbes and other TLR agonists is unclear. A number of studies have demonstrated that the PI3K/Akt pathway can play a critical role in positively regulating TLR-induced proinflammatory signaling and cytokine production in inflammatory cells in response to certain ligands (31, 42, 43). Indeed, Akt has been recently implicated as a positive regulator of proinflammatory responses to F. tularensis subsp. novicida (44). Other reports, however, have demonstrated that the PI3K/Akt pathway negatively regulates TLR-induced MAPK activation and proinflammatory cytokine production (43, 45–47). The PI3K/Akt pathway also has been shown to differentially regulate anti-inflammatory and proinflammatory cytokine responses of human PBMCs to different TLR ligands by inhibiting the activity of glycogen synthase kinase 3β (GSK3β) (48).

In this study, we provide evidence that the PI3K/Akt signaling pathway is a negative regulator of the early proinflammatory response to F. tularensis LVS infection. Pharmacological inhibition of PI3K during infection of mouse bone marrow-derived macrophages with LVS led to sustained and enhanced phosphorylation of the p38 and ERK1/2 MAPKs and enhanced expression of TNF and IL-6. The activation of PI3K by LVS was MyD88-dependent, but was surprisingly TLR2-independent. LVS infection also induced rapid expression of the MAPK phosphatase, MKP-1, in a PI3K-dependent manner, suggesting that the PI3K/Akt pathway attenuates the TLR2-dependent proinflammatory cytokine response to LVS by suppressing p38 MAPK and ERK1/2 activation, possibly via upregulation of MKP-1.

Materials and Methods

Reagents

Wortmannin, LY294002, SB203580, and PD98059 were purchased from Calbiochem (San Diego, CA) and dissolved in MeSO (Sigma-Aldrich, St. Louis, MO) before adding to cell cultures. Cytochalasin D was purchased from Sigma-Aldrich. Abs used include: rabbit polyclonal anti-Akt, rabbit monoclonal anti–phospho-Akt (Ser473) (clone 193H12), rabbit polyclonal anti-p44/42 MAPK, rabbit polyclonal anti–phospho-p44/42 MAPK (Thr202/Tyr204), rabbit polyclonal anti-p38 MAPK (Thr180/Tyr182) (clone 3D7), rabbit polyclonal anti-JNK, rabbit monoclonal anti–phospho-JNK (Thr183/Tyr185) (clone 98-F2), and monoclonal anti–phospho-NF-κB p65 (Ser536) (clone 93H1) (all from Cell Signaling Technology, Beverly, MA); and rabbit polyclonal anti-MKP-1 and HRP-linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Bacteria

F. tularensis subsp. holarctica LVS (ATCC 29684, American Type Culture Collection, Manassas, VA) was provided by Dr. Karen L. Elkins (Center for Biologies Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD). Inocula for infections were prepared by streaking a glycerol bacterial stock on chocolate agar. After 72 h, several bacterial colonies were harvested and spread evenly on chocolate agar. After 18–24 h, the lawn of bacterial growth was scraped from the plate and transferred to tryptic soy broth supplemented with 1% IsoVitalex (BD Microbiology Systems, Sparks, MD). Bacterial inocula prepared in this way were stored at 4°C and used within 1 wk of preparation, during which the titer of viable bacteria was stable. The titer of the inocula were determined by plating serial dilutions on chocolate agar plates (Remel, Lenexa, KS) on the day of infection. All protocols were approved by the Institutional Biosafety Committee at the University of Texas Health Science Center at San Antonio, San Antonio, TX.

Mice

C57BL/6 mice were purchased from the National Cancer Institute (NCI-Frederick Animal Production Area, Frederick, MD). Homozygous knock-out mice on a mixed C57BL/6 x129 genetic background and deficient in TLR1 (N4) (49), TLR2 (N4) (50), TLR4 (N4) (51), TLR6 (N4) (52), TLR9 (N8) (53), MyD88 (N4) (54), TIRAP/Mal (N1) (55), TRIF (N4) (56), and TRAM (N4) (57) were obtained under a materials transfer agreement from C57BL/6 mice were pre-exposed to the PI3K inhibitor wortmannin (100 nM) for 1 h and then exposed to LVS (MOI of 100) for 0.5–9 h. A, The relative levels of phospho-Akt (Ser473) and total Akt were assessed by Western blot, and the immunoreactive bands were detected with a Typhoon

FIGURE 1. Regulation of the proinflammatory cytokine response in LVS-infected macrophages by PI3K. Bone marrow-derived macrophages from C57BL/6 mice were pre-exposed to the PI3K inhibitor wortmannin (100 nM) for 1 h and then exposed to LVS (MOI of 100) for 0.5–9 h. A, The relative levels of phospho-Akt (Ser473) and total Akt were assessed by Western blot, and the immunoreactive bands were detected with a Typhoon 9400 as described in Materials and Methods. B, TNF, IL-6, and MCP-1 levels in quadruplicate culture supernatants from A were assessed by CBA. Representative results of four independent experiments are shown for A and two independent experiments for B. *p < 0.05 compared with control samples as determined by one-way ANOVA.
from Dr. Shizuo Akira (Osaka University, Osaka, Japan) via Dr. Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA). Mice were used at 12–18 wk of age. Mice were housed in ventilated cages under specific pathogen-free conditions and used in accordance with Institutional Biosafety Committee and Institutional Animal Use and Care Committee protocols at the University of Texas Health Science Center at San Antonio.

**Preparation of bone marrow-derived macrophages**

Bone marrow-derived macrophages were prepared according to Doherty and Coffman (58) with slight modifications. Bone marrow was harvested from the femurs and tibias of 12-wk-old mice. Cells were cultured for 8 d in complete RPMI 1640 medium (25 mM HEPES, 2 mM glutamine, 1% penicillin/streptomycin, 50 μM 2-ME, 10% heat-inactivated FBS) supplemented with 0.02 μg/ml murine GM-CSF (PeproTech, Rocky Hill, NJ); nonadherent cells were discarded before use.

**In vitro infections**

Macrophages were seeded in six-well culture plates at a density of 1.5 × 10⁶ cells/well and incubated in antibiotic-free RPMI 1640 medium (no GM-CSF) for 36 h at 37°C in 6% CO₂ before use. Cells were washed with antibiotic-free medium and infected with LVS (multiplicity of infection [MOI] of 100) in quadruplicate in 1.5 ml antibiotic-free medium for 0.5–9 h at 37°C in 6% CO₂. In some experiments, chemical inhibitors or diluent only were added to cultures 1 h before infection.

**Immunoblotting**

Whole-cell lysates were prepared by solubilizing cell pellets with PBS (pH 7.4) containing 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 0.2 mM Na₃VO₄. Lysates were cleared by centrifugation (16,000 × g) for 10 min and protein concentrations measured by Bradford protein assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein were resolved by 10% SDS-PAGE. Resolved proteins were transferred to PVDF membranes (Millipore Corporation, Bedford, MA), and the membranes were blocked for 1 h in TBS (pH 7.5) containing 0.1% Tween 20 and 5% nonfat dry milk and incubated overnight at 4°C with the appropriate primary Ab. Blocked membranes were incubated with an HRP-conjugated secondary Ab (Santa Cruz Biotechnology) for 1 h. ECL Plus reagent (GE Healthcare, Piscataway, NJ) was added for detection of immunoreactive bands, and blots were imaged with a Typhoon 9400 fluorescent imager (GE Healthcare). Quantification of immunoreactive bands was performed by volume analysis with local average background correction using ImageQuant software (GE Healthcare).

**Cytokine assays**

Supernatants harvested from cultured cells infected with LVS were frozen at −80°C until analysis. Samples were thawed and assayed using the BD Cytometric Bead Array (CBA) Mouse Inflammation kit (BD Pharmingen, San Diego, CA). Analysis of sample data was performed with BD Cytometric Bead Array Software (BD Pharmingen).

**Bacterial uptake assay**

WT C57BL/6 bone marrow-derived macrophages were seeded into 96-well plates (1.5 × 10⁶ cells/well) and cultured overnight. Cells were washed twice with sterile PBS, resuspended in complete RPMI 1640 medium, and treated with DMSO alone, cytochalasin D (5 μM) for 1 h, or Wortmannin (0.01 to 1 μM) for 1 h at 37°C. Cells were then infected with *F. tularensis* LVS (MOI 50 CFU/well). After a 2 h incubation to allow uptake of the bacteria, the infected cells were washed twice with sterile PBS and incubated for 30 min at 37°C in complete RPMI 1640 medium containing 50 μg/ml gentamicin to kill remaining extracellular bacteria. The infected cells were then washed twice with sterile PBS and lysed in 0.1% SDS. The number of cell-associated bacteria was determined by plating serial dilutions of the cell lysates on chocolate agar as described above. Assays were performed in triplicate for each treatment group.

**Statistical analysis**

All numerical data are presented as the mean ± SEM. Significance was determined using a Student *t* test or one-way ANOVA followed by the Holm-Sidak method of post hoc analysis for multiple group data. All statistical analysis was performed with SigmaStat 3.1 software (Systat Software, San Jose, CA).

**Results**

**Regulation of the proinflammatory cytokine response in LVS-infected macrophages by PI3K**

To assess the role of PI3K activation in regulating *F. tularensis* LVS-induced proinflammatory signaling, we pretreated macrophages with wortmannin, a widely used and highly specific inhibitor of PI3K (59). Macrophages from C57BL/6 mice were pre-exposed to wortmannin (100 nM) or vehicle alone for 1 h and then exposed to LVS (MOI of 100) for 0.5–9 h. The level of phosphorylated Akt was determined by immunoblotting as a measure of PI3K activity (Fig. 1A), and TNF, IL-6, and MCP-1 secretion was assessed by cytometric bead array assay (Fig. 1B). LVS infection stimulated a rapid and significant (5–7-fold) increase in Akt phosphorylation in vehicle pre-exposed macrophages, with peak phosphorylation occurring between 0.5 and 3 h postinfection (Fig. 1A; see below for quantification of Akt phosphorylation kinetics). As expected, wortmannin treatment markedly inhibited Akt phosphorylation. LVS infection induced macrophages to secrete TNF and IL-6 as expected (Fig. 1B), but secretion of these cytokines was significantly greater when the macrophages were pretreated with wortmannin (Fig. 1B). By contrast, secretion of MCP-1 was modestly inhibited by wortmannin treatment. A similar cytokine expression...
pattern was observed when macrophages were treated with another well-known inhibitor of PI3K activity, LY294002 (data not shown) (59). Treatment of uninfected macrophages with wortmannin or LY294002 had no effect on cytokine secretion. Titration of wortmannin in the macrophage cultures demonstrated that a concentration of 100 nM resulted in almost complete inhibition of PI3K activity and a marked enhancement of cytokine expression (Fig. 2A, 2B). Because previous studies have demonstrated that wortmannin can affect the phagocytosis of some pathogenic microorganisms (60–63), which might confound interpretation of our signaling studies, we examined the effects of PI3K inhibition on the uptake of LVS. Fig. 2C demonstrates that wortmannin had no significant effect on uptake of LVS, whereas cytochalasin D, a well-established inhibitor of phagocytosis (64), reduced uptake of LVS by ∼100-fold. These results are consistent with a recent study that demonstrated that PI3K is not required for the phagocytosis of *F. tularensis* subsp. novicida by macrophages (65). Taken together, these data indicate that LVS-induced activation of PI3K negatively regulates proinflammatory cytokine expression in infected macrophages.

**Regulation of proinflammatory signaling by PI3K in LVS-infected macrophages**

MAPK and NF-κB activation mediates LPS-induced TNF and IL-6 production in murine macrophages (66–70), and previous studies have shown that LVS infection can inhibit MAPK and NF-κB activation in macrophages by LPS (10). We therefore explored the role of PI3K in regulating MAPK and NF-κB activation in macrophage cultures infected with LVS. As shown in Fig. 3A and 3B, the levels of phosphorylated p38 MAPK induced by LVS were enhanced by treatment with wortmannin. Similar results were observed when PI3K was inhibited with LY294002 (57) (Fig. 3C and data not shown). Wortmannin treatment increased the levels of phosphorylated p38 MAPK in a dose-dependent manner with a significant increase first observed at a concentration of 100 nM (Fig. 3D, 3E), which is also the concentration at which the signal

![FIGURE 3. Regulation of proinflammatory signaling by PI3K in LVS-infected macrophages. Bone marrow-derived macrophages from C57BL/6 mice were pre-exposed to the PI3K inhibitors wortmannin (100 nM) (A, B) or LY294002 (50 μM) (C) for 1 h and then exposed to LVS (MOI of 100) for 0.5–9 h. For D and E, macrophages from C57BL/6 mice were pre-exposed to increasing concentrations of wortmannin (0 to 1 μM) for 1 h and then exposed to LVS (MOI of 100) for 1 h. A, The relative levels of total Akt, phospho-Akt (Ser473), phospho-p38 MAPK, and phospho-JNK were assessed by Western blot. B, Immunoreactive bands in A were detected with a Typhoon 9400 and quantified with ImageQuant software (GE Healthcare) as described in Materials and Methods. Quantified values for phosphorylated proteins were normalized to total Akt to account for variation in protein loading. A phosphorylation index was calculated for each phosphoprotein at each time point as the ratio of the normalized value for the specific phosphoprotein at time point X to the normalized value for that phosphoprotein at timepoint zero. C and D, The relative levels of total Akt, phospho-Akt (Ser473), and phospho-p38 MAPK were assessed by Western blot. E, Immunoreactive bands from D were quantified as described above, and a phosphorylation index was calculated for each phosphoprotein at each dose of wortmannin as the ratio of the normalized value for the specific phosphoprotein at dose X to the normalized value for that phosphoprotein in the absence of wortmannin (0 nM). Representative results of two independent experiments are shown for A–C. Representative results of three independent experiments are shown for D and E. (A, B) The relative levels of total Akt, phospho-Akt (Ser473), and phospho-p38 MAPK were assessed by Western blot. (B) Immunoreactive bands in A were detected with a Typhoon 9400 and quantified with ImageQuant software (GE Healthcare) as described in Materials and Methods. Quantified values for phosphorylated proteins were normalized to total Akt to account for variation in protein loading. A phosphorylation index was calculated for each phosphoprotein at each time point as the ratio of the normalized value for the specific phosphoprotein at time point X to the normalized value for that phosphoprotein at timepoint zero. C and D, The relative levels of total Akt, phospho-Akt (Ser473), and phospho-p38 MAPK were assessed by Western blot. E, Immunoreactive bands from D were quantified as described above, and a phosphorylation index was calculated for each phosphoprotein at each dose of wortmannin as the ratio of the normalized value for the specific phosphoprotein at dose X to the normalized value for that phosphoprotein in the absence of wortmannin (0 nM). Representative results of two independent experiments are shown for A–C. Representative results of three independent experiments are shown for D and E.
for phosphorylated Akt first disappeared (Figs. 2A, 3D, 3E) and at which enhancement in cytokine production first occurred (Fig. 2B). Inhibition of PI3K had no consistent effect on JNK or NF-κB p65 phosphorylation (Fig. 3A, 3B). To determine the extent to which the activation of p38 and ERK1/2 in response to LVS infection is responsible for TNF and IL-6 expression, macrophages were pre-exposed to the p38 inhibitor SB203580 or the MEK1/2 inhibitor PD98059 or both prior to LVS infection. Both p38 and MEK1/2 (and consequently ERK1/2) inhibition markedly decreased TNF levels, whereas simultaneous inhibition of both MAPKs completely suppressed cytokine production (Fig. 4A). Simultaneous inhibition of p38 MAPK and MEK1/2 also significantly suppressed IL-6 production (Fig. 4B). Inclusion of wortmannin in the cultures failed to relieve the inhibition of cytokine expression by the MAPK inhibitors (data not shown), demonstrating that the inhibition of PI3K cannot upregulate cytokine expression in LVS-infected macrophages in the absence of MAPK activation. This suggests that the enhanced and sustained activation of p38 and ERK1/2 by LVS when PI3K is inhibited by wortmannin (Fig. 3) is responsible for the significant increase in cytokine production (Figs. 1B, 2B). Production of MCP-1 was not suppressed by the MAPK inhibitors (Fig. 4C). Collectively, these data suggest that activation of the PI3K pathway by LVS infection represses proinflammatory cytokine production in macrophages by attenuating p38 and ERK1/2 activation.

The role of TLRs in LVS-induced proinflammatory signaling

Recent studies indicate that the induction of the innate inflammatory response of mice to LVS is mediated by TLR2 (11–13, 15, 24). Although both LVS and F. tularensis subsp. novicida clearly induce MAPK activation and activate Akt in macrophages (10, 44), the regulation of these pathways by TLRs has not been explored. Because we observed that PI3K negatively regulates LVS-induced proinflammatory signaling and cytokine production, we sought to clarify the role of TLRs in triggering these apparently opposing signaling responses. Macrophages from C57BL/6 (WT), TLR1<sup>−/−</sup>, TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, TLR6<sup>−/−</sup>, or TLR9<sup>−/−</sup> mice were exposed to LVS (MOI of 100) for 0.5–9 h, and cell lysates were analyzed by immunoblotting. Fig. 5A and 5B demonstrate that, whereas control macrophages responded as expected, LVS infection induced virtually no phosphorylation of MAPKs or NF-κB p65 in macrophages from TLR2<sup>−/−</sup> mice, with the exception that ERK1/2 phosphorylation was partially TLR2-independent. There was no difference in the pattern of LVS-stimulated phosphorylation of these proteins in macrophages from TLR1<sup>−/−</sup>, TLR4<sup>−/−</sup>, TLR6<sup>−/−</sup>, and TLR9<sup>−/−</sup> mice when compared with WT controls (data not shown). Surprisingly, the pattern of Akt phosphorylation induced by LVS infection in all of the TLR-deficient macrophages, including TLR2<sup>−/−</sup> macrophages, was similar to the pattern observed in WT macrophages (Fig. 5A, 5B and data not shown). TNF and IL-6 secretion was impaired in macrophages from TLR2<sup>−/−</sup> mice (Fig. 5C), but the extent of cytokine production was identical among macrophage cultures from WT, TLR1<sup>−/−</sup>, TLR4<sup>−/−</sup>, TLR6<sup>−/−</sup>, and TLR9<sup>−/−</sup> mice (data not shown). Thus, activation of NF-κB and MAPKs and, as reported previously (11–13, 15, 24), proinflammatory cytokine expression in macrophages all appear to depend largely on TLR2, whereas activation of PI3K is independent of TLR2 (and TLR4 and TLR9).

The role of TLR adaptors in LVS-induced activation of PI3K

Each TLR signals through one or more adaptor proteins: TLRs 2, 5, 7–9, and 11–13 signal through the classic MyD88-dependent pathway, TLR4 can signal via the MyD88-dependent pathway and through a MyD88-independent pathway requiring the adaptors TRIF and TRAM, and TLR3 signals via TRIF. Among TLRs

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** The role of MAPKs in regulating proinflammatory cytokine production in LVS-infected macrophages. Bone marrow-derived macrophages from C57BL/6 mice were pre-exposed to the p38 MAPK inhibitor SB203580 (25 μM) or the MEK1/2 inhibitor PD98059 (25 μM) or both for 1 h prior to LVS infection. The levels of TNF, IL-6, and MCP-1 in quadruplicate culture supernatants were assessed by CBA. Representative results of two independent experiments are shown in A–C. *p < 0.05 compared with control samples as determined by one-way ANOVA.
that signal via MyD88, TLR2 and TLR4 also require the adaptor TIRAP/Mal (17, 22). To further explore activation of the PI3K pathway, we assessed responses to LVS infection in macrophages from TLR adaptor-deficient mice. Macrophages from WT, MyD88^2/2, TIRAP/Mal^2/2, TRIF^2/2, or TRAM^2/2 mice were exposed to LVS (MOI of 100) for 0.5–9 h, and the cell lysates were analyzed by immunoblotting. As expected for TLR2-dependent responses, phosphorylation of NF-κB p65, ERK1/2, p38 MAPK, and JNK were severely attenuated in macrophages from MyD88-deficient and TIRAP/Mal-deficient mice (Fig. 6 A, 6B). TNF and IL-6 secretion were also significantly depressed in MyD88- and TIRAP/Mal-deficient macrophages (Fig. 6 C).

LVS did not induce Akt phosphorylation in macrophages from MyD88^2/2 mice (Fig. 6 A, 6B), but there was weak Akt activation in macrophages from MyD88^−/− mice; Akt activation was completely independent of TRIF and TRAM (data not shown). Although PI3K also can be activated by IL-1β and IL-18, and Francisella can induce the expression of these cytokines (12, 71), neutralizing Abs to IL-1β and IL-18 had no effect on LVS-induced Akt phosphorylation in WT macrophages (data not shown). Thus, activation of PI3K by LVS infection is independent of TRIF, TLR2, TLR3 (not affected by TRIF deficiency), TLR4, TLR6, TLR9, IL-1β, and IL-18, but is dependent on MyD88.

LVS infection induces MKP-1 expression in primary macrophages in a TLR2/MyD88-dependent manner

MKP-1 has been established as a key regulator of innate immune responses because of its ability to dampen TLR-induced MAPK activation (72–76). Because PI3K inhibition enhanced and prolonged LVS-induced MAPK phosphorylation, we asked whether LVS infection could stimulate MKP-1 expression and whether such expression is regulated by the PI3K pathway. Macrophages were pre-exposed to wortmannin for 1 h and then exposed to LVS (MOI of 100) for 0.5–9 h as before, and MKP-1 expression was analyzed by immunoblotting. As expected for TRIF- and TRAM-dependent responses, phosphorylation of NF-κB p65, ERK1/2, and JNK were severely attenuated in macrophages from MyD88-deficient mice (Fig. 7 A, 7B). Pretreatment with wortmannin markedly decreased MKP-1 expression; as described earlier, the levels of phosphorylated p38 and ERK1/2 were enhanced and prolonged upon inhibition of PI3K by wortmannin (Fig. 3 A, 3B). We also asked to what extent the TLR2 signaling pathway is required for induction of MKP-1 expression by LVS. LVS-induced MKP-1 expression was markedly reduced in macrophages from TLR2^2/2 mice (Fig. 7 B) and in MyD88^−/− mice (Fig. 7 C). These data demonstrate that MKP-1 expression is induced by LVS infection in a TLR2-dependent and PI3K-dependent manner and suggest that PI3K activation by LVS may suppress MAPK activation in macrophages in part by induction of MKP-1 expression (see proposed model in Fig. 8).

Discussion

The inability of the innate immune response to prevent lethal dissemination of F. tularensis is likely due, in part, to attenuated and/or delayed proinflammatory cytokine production in the infected host early postinfection. For example, proinflammatory
cytokine responses are significantly delayed in the lungs of mice infected intranasally with *F. tularensis* (7, 8) and *F. tularensis* infection of macrophages and dendritic cells in vitro has been shown to suppress secretion of TNF and IL-12, respectively (10, 77, 78). Little is known, however, of the mechanisms by which *F. tularensis* infection can suppress host responses. In the current study, we provide evidence for a model in which *F. tularensis* LVS induces a PI3K-dependent negative regulatory pathway that restrains TLR2-mediated p38 and ERK1/2 MAPK signaling and proinflammatory cytokine production (Fig. 8). The p38 and ERK1/2 MAPKs are rapidly activated following LVS infection in a TLR2- and MyD88-dependent manner, but levels of phospho-NF-κB p65 (Ser536), phospho-ERK1/2, phospho-p38 MAPK, and phospho-JNK were assessed by Western blot as described in the Materials and Methods. B, Immunoreactive bands in A were detected with a Typhoon 9400 and quantified with ImageQuant software (GE Healthcare) as described in Materials and Methods. Phosphorylation Indices were calculated as described in the legend to Fig. 3B and plotted versus time postinfection. C, TNF and IL-6 levels in quadruplicate culture supernatants from A were assessed by CBA. Representative results of two independent experiments are shown in A–C. *p < 0.05 compared with control samples as determined by one-way ANOVA.

**FIGURE 6.** The role of TLR adaptors in LVS-induced activation of PI3K. Bone marrow-derived macrophages from C57BL/6 (WT), MyD88−/−, and TIRAP/Mal−/− mice were exposed to LVS (MOI of 100) for 0.5–9 h. A, The levels of total Akt, phospho-Akt, phospho-NF-κB p65 (Ser536), phospho-ERK1/2, phospho-p38 MAPK, and phospho-JNK were assessed by Western blot as described in the Materials and Methods. B, Immunoreactive bands in A were detected with a Typhoon 9400 and quantified with ImageQuant software (GE Healthcare) as described in Materials and Methods. Phosphorylation Indices were calculated as described in the legend to Fig. 3B and plotted versus time postinfection. C, TNF and IL-6 levels in quadruplicate culture supernatants from A were assessed by CBA. Representative results of two independent experiments are shown in A–C. *p < 0.05 compared with control samples as determined by one-way ANOVA.
Our findings with LVS contrast with the results of recent studies of macrophages infected with *F. tularensis* subsp. novicida (44, 82). Those studies found that pharmacological inhibition of the PI3K/Akt pathway during infection with *F. tularensis* subsp. novicida resulted in inhibition of proinflammatory cytokine expression. The reason for these discrepant findings is not known. One possibility is that there are as yet undetermined differences in the manner in which LVS and *F. tularensis* subsp. novicida interact with macrophages that result in important differences in the macrophage response. Indeed, one recent study demonstrated that 60% fewer LVS bacteria associate with human monocyte-derived macrophages compared with *F. tularensis* subsp. novicida (83), and another demonstrated that LVS has a 100-fold greater growth rate in rat macrophages (84). Moreover, we have observed that proinflammatory cytokine expression by macrophages is 10-fold greater from macrophages infected with *F. tularensis* subsp. novicida compared with those infected with an equivalent amount of LVS (I.R. Morris and M.T. Berton, unpublished observations).

It has recently been shown that the in vitro growth conditions, including the nature of the growth medium, can dramatically affect the virulence and immunostimulatory properties of *F. tularensis* (85). Thus, multiple factors may account for differences observed among studies of host cell signaling responses by the various *Francisella* species.

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It is well established that MKPs play a key role in the feedback control of proinflammatory signaling and cytokine production via PI3K during LVS infection of macrophages. LVS activates proinflammatory signaling by binding to TLR2 on the surface of macrophages. In parallel, LVS infection also results in activation of PI3K through a TLR2-independent but MyD88-dependent pathway. In conjunction with TLR2-dependent activation of NF-κB and MAPKs, PI3K activation results in induction of MKP-1 expression. MKP-1 can then inactivate MAPKs by dephosphorylation, resulting in suppression of TLR2-dependent proinflammatory gene expression.
MAPK activation in response to TLR ligands (73–76). For example, LPS was shown to induce rapid MKP-1 expression in alveolar macrophages, which correlated with the downregulation of p38 MAPK, JNK and ERK1/2 phosphorylation, and peritoneal macrophages from MKP-1−/− mice exhibited enhanced p38 MAPK, JNK, and ERK1/2 activation in response to LPS (87). Salojin et al. (75) also observed enhanced p38 and ERK1/2 phosphorylation in response to LPS in peritoneal macrophages from MKP-1−/− mice. Whereas the evidence that MKP-1 deactivates p38 MAPK and JNK is consistent and convincing, its role in restraining ERK1/2 is less certain. Several recent studies demonstrated that exposure of macrophages from MKP-1−/− mice to LPS (73, 74, 76), peptidoglycan, or lipoteichoic acid (88) resulted in prolonged p38 MAPK activation as well as enhanced TNF and IL-6 production; however, there were no differences in the extent of ERK1/2 activation compared with WT macrophages. Yet, rigorous studies have clearly demonstrated that MKP-1 can dephosphorylate ERK2 (89, 90). These disparate findings may be due to differences in cell type and/or experimental conditions. Because multiple MAPK phosphatases can dephosphorylate ERK1/2, such as phosphatase of activated cells 1 and MKP-2 (89), it is possible that additional MKPs participate with MKP-1 to inactivate ERK1/2 in our system. Further studies are needed to determine the extent to which MKP-1 and other MKPs restrain LVS-induced ERK1/2 activation.

Consistent with its function as a negative feedback regulator of MAPKs, stimulation of MKP-1 expression in response to TLR ligands appears to be dependent on p38 MAPK activity (91). Thus, our observation that LVS-induced MKP-1 expression was largely dependent on TLR2 was not surprising given that p38 activation was completely TLR2-dependent. Interestingly, we observed that inhibition of p38 MAPK also markedly reduced MKP-1 expression in LVS-infected macrophages. Thus, optimal induction of MKP-1 expression by LVS appears to require both TLR2-dependent activation of p38 and TLR2-independent activation of MKP-1. A role for PI3K in the induction of MKP-1 expression has also been reported recently in vascular smooth muscle cells stimulated with TLR2 ligands (92). Future studies will determine if both PI3K and MKP-1 pathways may be responsible for the ability of *F. tularensis* to suppress or delay the expression of critical proinflammatory cytokines in vivo.

It was surprising that activation of the PI3K pathway by LVS infection was TLR2-independent; LVS activates the innate immune response largely through TLR2, and TLR2 ligands have been reported to stimulate PI3K activation (93–95). Activation of this pathway, however, was completely MyD88-dependent, which suggests that LVS may also signal host cells via a TLR other than TLR2, although neither TLR3, TLR4, nor TLR9 appear to be required based on our results. This possibility is supported by recent studies that demonstrated that MyD88 is not required for TLR2-dependent PI3K activation in macrophages (96), but is required for activation of PI3K by other TLRs (33–35). It is also possible that LVS induces the secretion of a cytokine or other soluble factor in the macrophage cultures that then activates the PI3K pathway through a TLR2-independent, MyD88-dependent pathway. In that regard, the neutralization of IL-1β or IL-18, two cytokines known to signal via MyD88, had no effect on the activation of PI3K in the LVS-infected cultures (data not shown). Whatever the nature of this TLR2-independent pathway, it does not appear to play a significant role in the positive regulation of the proinflammatory cytokine response to LVS infection because there was virtually no MAPK phosphorylation, NF-κB activation, or cytokine production in macrophages from TLR2−/− mice. Thus, it is tempting to speculate that there is a component of LVS, distinct from the ligands for TLR2, that interacts with an additional TLR/PRR to stimulate PI3K and oppose the TLR2-mediated proinflammatory response.

In summary, we observed that early (<8 h) during LVS infection of macrophages, activation of PI3K suppresses MAPK activation and proinflammatory cytokine production. The suppression correlates with robust induction of MKP-1 expression, a phosphatase known to negatively regulate p38 MAPK and ERK1/2 activity. Whereas MAPK phosphorylation and cytokine production are entirely TLR2-dependent, activation of PI3K is TLR2-independent, but MyD88-dependent. We propose that LVS engages an additional MyD88-dependent PRR to activate this PI3K-mediated inhibitory pathway. This may represent a strategy that *F. tularensis* evolved to thwart host responses during the early stages of infection and thereby enhance virulence.

Acknowledgments
We thank Leslie Linehan for expert animal care. We also thank Bijaya Parida for assisting with preparation of bone marrow-derived macrophages and Dr. Shizuo Akira and Dr. Doug Golenbock for providing breeding pairs of the TLR-deficient and TLR adaptor-deficient mice.

Disclosures
The authors have no financial conflicts of interest.

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